J Assist Reprod Genet (2010) 27:233–238
DOI 10.1007/s10815-010-9411-x

FERTILITY PRESERVATION

Mouse oocyte vitrification: the effects of two methods on maturing germinal vesicle breakdown oocytes

Somayeh Khosravi-Farsani · Aligholi Sobhani · Fardin Amidi · Reza Mahmoudi

Received: 25 November 2009 / Accepted: 21 March 2010 / Published online: 21 April 2010

Abstract
Purpose Evaluation of viability and subsequent developmental ability of mouse germinal vesicle breakdown oocytes vitrified in conventional straws.
Methods Oocytes with compact cumulus cells were cultured for 3 h in TCM199 medium GVBD and vitrified by two methods: the step-wise and single-step. After vitrification, the oocytes were thawed, and subjected to in vitro maturation and in vitro fertilization. Oocyte survival (post-thaw) was assessed by morphological appearance and staining, using propidium iodide (PI)/Hoechst 33342. The oocyte maturation and fertilization rates were examined in vitro.
Results In the single-step method the rates of post thaw survival, maturation to metaphase II and cleavage (2-cell embryos) were 58.68%, 56.41% and 38.63%, respectively. In the step-wise method, the corresponding rates were 81.75%, 68.59% and 51.80%, respectively.
Conclusion Vitrification of mouse germinal vesicle breakdown oocytes by the step-wise method had the advantage of maintaining the viability and subsequent production of 2-cell embryos. In comparison with that in unvitrified control oocytes, the development of MII oocytes to 2-cell embryos was impaired following vitrification.

Keywords Cryopreservation · Cumulus cell · In vitro maturation · Oocyte · Viability

Capsule Vitrification of mouse cumulus germinal vesicle breakdown oocyte complexes by a step-wise manner had the advantage of maintaining the viability and subsequent production of 2-cell embryos.

S. Khosravi-Farsani · A. Sobhani · F. Amidi
Department of Anat., Faculty of Medicine,
Tehran University of Medical Sciences,
Tehran, Iran

R. Mahmoudi (✉)
Dept. of Anat., Faculty of Medicine,
Yasuj University of Medical Sciences,
Yasuj, Iran
e-mail: rmahmoudi40@yahoo.com

Introduction

Cryopreservation of oocytes has become an essential part of assisted reproductive technology (ART), particularly in vitro fertilization and embryo transfer (IVF-ET). Being first introduced in 1986, human oocyte cryopreservation has a relatively short history [1]. Oocytes can be cryopreserved using slow cooling or vitrification. Vitrification is an alternative to traditional freezing methods (slow freezing) to avoid chilling injury and ice crystal formation [2, 3]. Kuleshova *et al.* reported the first birth from vitrified human oocytes [4]. At present, various protocols are used for freezing oocytes, but these protocols have been shown to be unsatisfactory and appear to need more improvement of the methods. The survival of mammalian oocytes and embryos after cryopreservation varies with the stage of maturation and development [5, 6] and the cell cycle stage during meiosis affects the outcome due to varying sensitivity to cooling procedures [7]. Until recently, all efforts have focused on the cryopreservation of mature oocytes (MII), but at this stage, meiotic spindle microtubules and the microfilament may be damaged by cooling temperature and exposure to cryoprotectants [8-10]. Additionaly, failure of the meiotic spindles could lead to chromosomal dispersion and high incidence of aneuploidy or polyploidy [8, 11]. Some studies

have described irreversible structural damage to the oocyte membrane or impaired intercellular communication between the oocyte and the cumulus cells after cryopreservation of germinal vesicle (GV) oocytes [12]. Therefore, choosing an intermediate stage, such as germinal vesicle breakdown (GVBD), may circumvent some of the problems associated with the cryopreservation of GV and MII oocytes. Since 1998, most studies in this connection have focused on the maturing oocytes but except some research on bovine GVBD oocyte [6, 13-15] few studies have been carried out on GVBD oocyte stage. Mouse oocyte cryopreservation is important as it can provide preliminary data applicable to human oocyte cryopreservation. Furthermore, studies on mouse oocyte cryopreservation can provide basic cryobiological data. In this manner, it has been suggested that vitrification may be less traumatic to the meiotic spindle than slow freezing and may also have fewer adverse effects on cell physiology [16]. However, more studies including animal research are still required to elucidate the issue [17]. Cumulus cells are thought to protect the oocyte against cell damage during cryopreservation, although the mechanisms involved have yet to be elucidated [18]. The damage occurring to the cumulus cells surrounding the oocytes could affect the success of immature oocyte cryopreservation [19]. According to these indications, the success of immature oocyte cryopreservation could depend on the capacity of cumulus oocyte complexes (COCs) to preserve its structural and functional integrity as a whole. There are few studies about cumulus cells viability after vitrification to improve cryopreservation procedures [7]. Hence, in this study we analyzed the effects of vitrification on GVBD oocyte and cumulus cells simultaneously. Also, as step-wise vitrification has had better outcome in other developmental stages [20, 21], the current study was undertaken to evaluate the effects of step-wise and single-step cooling on intact GVBD oocytes competence following vitrification.

Materials and methods

All the chemicals used in this project, except TCM media which was obtained from Gibco (Gibco, UK), were purchased from sigma-Aldrich chemie.

1. Animals and collection of GV oocytes Female NMRI (4 to 8 weeks old) mice were kept under controlled condition (14 h light, 10 h dark) and were stimulated by i.p. injection of 10 IU of pregnant mare serum gonadotropin (PMSG). After 48 h, the animals were killed by cervical dislocation; the ovaries were removed and transferred into a holding medium, which consisted of M199 medium supplemented with 10% fetal bovine serum (FBS). GV oocytes were obtained by puncturing of antral follicles in a holding medium. In all experiments, only full-grown cumulus oocyte complexes (COCs) with compact cumulus cell layer were used. The animal care was conducted in accordance with the institutional guidelines of Tehran Medical Sciences University and the National Institutes of Health (NIH) guidelines for the care and use of laboratory animals.
2. In vitro maturation COCs were washed three times in the holding medium (TCM199 supplemented with 10% FBS) and placed in 100 µl microdrops of maturation medium covered with mineral oil, remaining for 3-4 h at 37°C in an atmosphere of 5% CO2 in humid air. The maturation medium (TCM 199) was supplemented with 10 %FBS, 0.23 mM sodium pyruvate, 10 ng/mL epidermal growth factor, 100 mIU FSH (GONAL–*F* serono), 75 µg/ml penicillin G-K salts, and 50 µg/ml streptomycin sulfate.
3. Vitrification of COCs The maturing GVBD oocytes with cumulus cells were vitrified as described by Aono *et al.* [20] with some modifications. The holding medium used for handling oocytes during vitrification was TCM199 containing 10% FBS. All vitrification solutions were prepared using this holding medium. The manipulation of oocytes and vitrification process were performed at room temperature (25◦C). The oocytes of step-wise group were exposed to vitrification solution of %2.5 EG for 5 min, %5 EG for 3 min and %10 EG for 2 min, before exposing them to the final vitrification solution (%30 EG +0.5 M sucrose) for 40–60 s. In the single-step group, the oocytes were exposed to final vitrification solution for 1 min. Subsequently, they were loaded in 0.25 ml straws in the middle column of the vitrifing solution separated by air bubbles from about 20 µl of the same medium on each side. Ten oocytes were loaded in each straw and immediately dipped vertically in liquid nitrogen. The straws were stored for 7 days in liquid nitrogen.
4. Thawing The straws were thawed in air for 10 s and immediately plunged into a water bath at 37◦C for 10 s. Thawing was carried out in four steps using sucrose solution in a holding medium containing %10 FBS at 37 ◦C, and afterwards the oocytes were transferred to the decreasing concentrations of sucrose (0.5, 0.2, 0.1 and 0.05 M**)** at 1 min intervals. GVBD oocytes were washed three times at 37◦C in maturation medium before being transferred for the maturation protocol. After thawing, GVBD oocytes were matured for additional 21 h. to fulfill the 24 h maturation requirement.
5. Assessment of oocyte viability Oocyte survival was evaluated morphologically based on the integrity of the oolema and zona pellucida; Oocytes were also assessed

for viability based on oolema integrity by propidium iodide (PI) and Hoechst. For this purpose, oocytes were stained with PI (10 μg/ml) and Hoechst 33342 (10 μg/ml) for 10 min, washed, and then observed under a fluorescence microscope. The dead cells showed red fluorescence (PI-positive) for disruption of cellular membrane and the viable cells showed blue fluorescence without red fluorescence (PI-negative) for the intact cell membrane [22].

6. Assessment of cumulus cells viability Cumulus oocyte complexes in the control group and after step-wise and single-step vitrification were held in 0.1% hyaluronidase medium. COCs were denuded by mechanically pipetting with a fine diameter Pasteur pipette. Cumulus cells were washed in the holding medium and transferred into separate 30 μl droplet of maturation medium and 30 μl of 0.4% trypan blue solution was added. The viable cumulus cells were counted by Neubauer counting. The dead cells were dark for disruption of cellular membrane.

7. In vitro fertilization Male NMRI mice aged 10 to 12 weeks old were killed by cervical dislocation, and caudal epididymal spermatozoae were released by puncturing into Hams-F10 medium, supplemented with 4 mg/ml bovine serum albumin and kept for 1.5–2 h at 37°C to allow capacitation. The control as well as frozen-thawed oocytes were transferred to plates containing 200 μl of T6 medium (Royan. Iran), supplemented with 15 mg/ml bovine serum albumin (10–12 oocytes in each drop), and incubated until insemination. Of the released sperms a concentration of 1-2×10^6 cells/ml was added to the insemination plates, and the mixture was further incubated at 37°C in an atmosphere of 5% CO2 in air for 5 h. Oocytes were then transferred through three droplets of T6 medium supplemented with 4 mg/ml BSA under mineral oil and incubated in the final droplet for a further 15 h at 37°C, in 5% CO2 atmosphere. At this point, the cells were assessed for normality and progression to the 2-cell embryos.

Experimental design

Experiment 1 After obtaining GVBD, the oocytes were exposed to the single-step or step-wise vitrification procedure. The first experiment was designed to evaluate GVBD oocyte survival after vitrification. Non-vitrified and the control oocytes were cultured in the same way. Oocyte viability was evaluated simultaneously by Hoechst 33342 and PI staining for morphology appearance.

Experiment 2 As in experiment 1, cumulus cells were evaluated by staining with trypan blue and the viable cumulus cells were counted by Neubauer counting.

Experiment 3 After step-wise and single-step vitrification and thawing, the round-shaped oocytes with intact plasma membranes (Fig. 1) and the control group were subjected to maturation and fertilization in vitro. The rates of maturation and 2-cell embryos formation were compared between the groups.

Statistical analysis

The data were analyzed, using ANOVA. A P-Value<0.05 was considered as significant.

Results

Experiment 1 As shown in Fig. 1, the viability assessment of oocytes based on oolema integrity by Hoechst and PI showed red fluorescence for the dead cells due to disruption of cellular membrane (PI-positive) and blue fluorescence (Fig. 1) for the viable cells because of the intact cell membrane (PI-negative). The survival rate in the step-wise group (81.75%) was significantly (P<0.05) higher than that in the single-step group (58.68%) (Table 1).

Experiment 2 The dead cumulus cells showed blue stain after the single-step and step-wise vitrifica-

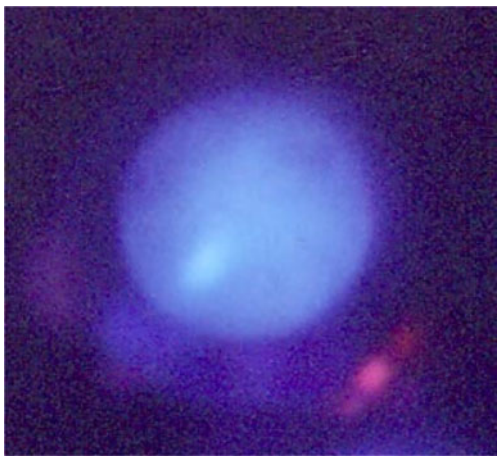

Fig. 1 GVBD oocyte after staining with Hoechst/ PI. Viable oocyte shows blue fluorescence. There is a dead cumulus cell that shows red fluorescence

Table 1 GVBD oocytes and survival rates in experimental groups

Groups	No. of GVBD oocytes	No. of GVBD oocytes vitrified	No. (%) of oocytes survived
Control (Non vitrified)	109	–	–
Step-wise	148	148	121 (81.75)[a]
Single-step	133	133	78 (58.68) [b]

GVBD Germinal Vesicle Breakdown

The survival rate of GVBD oocytes after single or step-wise vitrification was assessed based on morphology and HO/PI staining

[ab] The values in same column with different letters differ significantly, ($P<0.05$)

tion because of disruption of cellular membrane as in the control group but the viable cells were stainless in all groups. The survival rate of cumulus cells in the step-wise and single-step groups was 89.69% and 72.32%, respectively. The rate of viable cumulus cells after vitrification in step-wise group was not significantly different ($P>0.05$) from that of the control group (95.13%) (Fig. 2).

Experiment 3 As shown in Table 2, 68.59% and 56.41% of oocytes in step-wise and single-step groups were respectively developed to the MII and were significantly lower than that of the control group (84..40). The number of 2-cell embryos formation in the step-wise group was significantly higher than that of the single-step group (51.80%, 38.63%, respectively) and they were significantly lower ($P<0.05$) than those of the control group (73.91%).

Discussion

In this study, we showed that cryopreservation by vitrification enabled mouse GVBD oocytes to survive, mature, fertilize and develop to two-cells. Successful cryopreservation of GVBD oocytes has been reported in a few studies, particularly those on in bovine and calf [14, 15]. Since vitrification is a nonequilibrium cryopreservation method that needs a relatively high concentration of cryoprotectants, a step-wise addition of cryoprotectants may reduce the toxic effects of cryoprotectants and be considered to minimize damage due to extreme cell–volume expansion [23, 24]. In fact, for vitrification of GVBD mouse derived from IVM, a four-step exposure to the cryoprotectants showed less damage compared with the damage caused by the single-step procedure. Despite improvement in the development of GVBD oocytes to the MII and production of viable 2-cell embryos by the step-wise exposure, both maturation and cleavage rates were still low in comparison with that in the non-vitrified control. The importance of survival is especially critical when a limited number of cells are available for cryopreservation [25]. Our results in survival rate after the step-wise vitrification are consistent with the report of Men *et al*. that showed vitrification of bovine GVBD oocytes after a two-step vitrification (81.75% versus 79.59%). In fact, in vitrification of GVBD mouse derived from IVM, the four-step exposure to the cryoprotectants showed less damage compared with that in single-step procedure. The four-step vitrification in our study might be responsible for higher maturation rate than the rate reported by Men *et al.* [14] who used two-step vitrification. Other factors accounting for such a difference might be the different size of oocyte in bovine and mouse and the different CPAs (Cryoprotectant agents) in vitrification. Mahmoudi *et al.* [26] reported that intact immature mouse oocytes had a higher developmental competence than denuded oocytes. Hurt *et al.* [27] reported that vitrification with Ethylene Glycol (EG) would lead to expansion of cumulus cells probably due to damage of the gap junctions between the cumulus cells and oocytes. In this study, as Cetin & Bastan [28] stated, contrary to Hurt

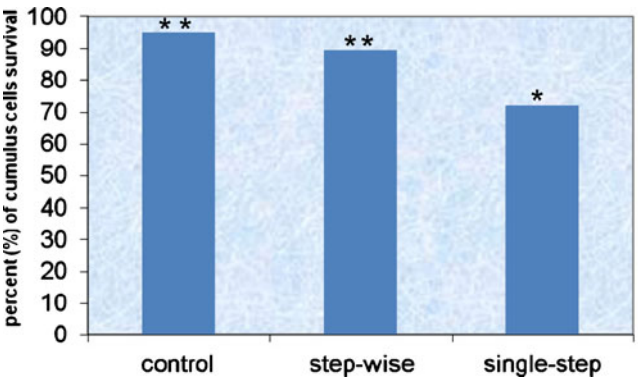

Fig. 2 Survival rates of cumulus cells of GVBD oocytes following vitrification by the single-step or step-wise methods. Viability was assessed based on trypan blue staining. *$P<0.05$ was considered as significant. $^{*}P<0.05$; $^{**}P>0.05$

Table 2 Development rates of GVBD oocytes to the MII and 2-cell embryos in vitro in the experimental and in control groups

Groups	No. of oocytes cultured	No. (%) of oocytes matured to MII	No. (%) of 2-cell embryos formation
Control (Non vitrified)	109	92 (84.40)	79 (73.91)[a]
Step-wise	121	83 (68.59)	62 (51.80)[b]
Single-step	78	44 (56.41)	30 (38.63)[c]

[a-c] Values in same column with different letters differ significantly, ($P<0.05$). (4 Replicate)

et al., in the step-wise vitrification with EG, distribution of cumulus cells did not occur after dissolution and the oocytes in the step-wise group preserved their conection with cumulus cells and less percent of cumulus cells were dead. So, there was not a significant deference in viability of cumulus cells between the control and step-wise groups. Bovine oocytes at the GVBD stage have been said to be more resistant to cooling than GV or MII oocytes [29]. However, when cryopreservation was attempted, Men *et al.* [14] observed that a significantly higher proportion of cleaved bovine embryos from vitrified MII oocytes than those derived from vitrified GVBD oocytes developed into blastocysts. Aono *et al*. [21] showed high rates of survival, maturation and 2-cell embryos formation (98.6%, 92.6 % and 79.4% respectively). These were obtained when mouse GV oocytes were ultra rapidly vitrified by increasing steps for preequilibration to CPAs. These rates are higher than what we found in vitrification of GVBD oocytes. The lower embryonic development in our experiment using GVBD stage oocytes compared with the results reported by Aono and co-workers might suggest that a four-step protocol be not as well as 10-step protocol. The cleavage rate we obtained after the step-wise vitrification was higher than that reported by Jee *et al.* who used GV oocytes in step-wise vitrification [30]. Comparison of the results between different developmental stages could help to choose the best oocyte for vitrification. Our results in this study are limited just to 2-cell formation, but some other studies are necessary to analyze developmental capacity up to blastocyst and after embryo transfer (ET). It might help to improve studies on oocyte vitrification. The reasons for the high sensitive nature of GVBD oocytes are unknown. In bovine and mammalian oocytes, active transcription and translation occur at GVBD and later stages of meiotic maturation [31, 32]. Therefore, in addition to the detrimental effects on the cytology, the biochemical process within the oocytes may also be affected by cryopreservation. The impaired biochemical process will negatively influence the cytoplasmic maturation of oocytes.

Cumulus cells appear to be important in maturation of oocyte [26]. It has been reported that the removal of cumulus cells before vitrification may induce a significant decrease in maturation promoting factor (MPF) and mitogen-activated protein kinase (MAPK) levels that are necessary for normal progression of the meiotic cycle and developmental competence [7]. In our study cumulus cells maintained their integrity after step-wise vitrification. The reduced viability and meiotic competence observed in the single-step group could result from an extensive loss of cumulus cell and reduction in the gap-junction communication between oocytes and cumulus cells. Other studies are necessary to investigate the effects of vitrification on molecules in oocytes before and after cryopreservation.

Conclusion

This study demonstrated that the four-step vitrification protocol was superior to the single–step protocol and it could maintain cumulus cell integrity that is necessary for viability, maturation and developmental competence of oocytes. Similar to other developmental stages the developmental rates after GVBD oocytes vitrification were lower in compare to control group. Further researches will help to clarify the cellular and molecular mechanisms of cryopreservation induced injury.

Acknowledgement This project financially supported by research deputy of Tehran University of Medical Sciences and Health services grant No. 85-04-30-4669. So, we would like to thank for their cooperation.

References

1. Chen C. Pregnancy after human oocyte cryopreservation. Lancet. 1986;1:884–6.
2. Rall WF, Fahy GM. Ice-free cryopreservation of mouse embryos at _196 8C by vitrification. Nature. 1985;313:573–5.
3. Gupta MK, Uhm SJ, Lee HT. Cryopreservation of immature and in vitro matured porcine oocytes by solid surface vitrification. Theriogenology. 2007;67:238–48.
4. Kuleshova L, Gianoroli L, Magli C, Ferraretti A, Trounson A. Birth following vitrification of small number of human oocytes. Hum Reprod. 1999;14:3077–9.
5. Pedro PB, Yokoyama E, Zhu SE, Yoshida N, Valdez Jr DM, Tanaka M, et al. Permeability of mouse oocytes and embryos at various developmental stages to five cryoprotectants. J Reprod Dev. 2005;51:235–46.
6. Magnusson V, Feitosa WB, Goissis MD, Yamada C, Tavares LM, D'Avila Assumpção ME, et al. Bovine oocyte vitrification: effect of ethylene glycol concentrations and meiotic stages. Anim Reprod Sci. 2008;106:265–73.

7. Bogliolo L, Ariu F, Fois S, Rosati I, Zedda MT, Leoni G, et al. Morphological and biochemical analysis of immature ovine oocytes vitrified with or without cumulus cells. Theriogenology. 2007;68:1138–49.
8. Zenses MT, Bielecki R, Casper R, Leibo SP. Effects of chilling to 0_C on the morphology of meiotic spindles in human metaphase II oocytes. Fertil Steril. 2001;75:769–77.
9. Huang JY, Chen HY, Tan SL, Chian RC. Effects of osmotic stress and cryoprotectant toxicity on mouse oocyte fertilization and subsequent embryonic development in vitro. Cell Preserv Tech. 2006;4:149–60.
10. Hyttel P, Vajta G, Callesen H. Vitrification of bovine oocyteswith the open pulled straw method: ultrastructural consequences. Mol Reprod Dev. 2000;56:80–8.
11. Huang JY, Chen HY, Park JY, Tan SL, Chian RC. Comparison of spindle and chromosome configuration in in vitro–and in vivo-matured mouse oocytes after vitrification. Fertil Steril. 2008;90:1424–32.
12. Arav A, Zeron Y, Leslie SB, Behboodi E, Anderson GB, Crowe JH. Phase transition temperature and chilling sensitivity of bovine oocytes. Cryobiology. 1996;33:589–99.
13. Hochi S, Ito K, Hirabayashi M, Ueda M, Kimura K, Hanada A. Effect of nuclear stages during IVM on the survival of vitrified-warmed bovine oocytes. Theriogenology. 1998;49:787–96.
14. Men H, Monson RL, Rutledge JJ. Effect of meiotic stages and maturation protocols on bovine oocyte's resistance to cryopreservation. Theriogenology. 2002;57:1095–103.
15. Albarracin JL, Morato R, Izquierdo D, Mogas T. Vitrification of calf oocytes: effects of maturation stage and prematuration treatment on the nuclear and cytoskeletal components of oocytes and their subsequent development. Mol Reprod Dev. 2005;72: 239–49.
16. Valojerdi MR, Salehnia M. Developmental potential and ultra-structural injuries of metaphase II (MII) mouse oocytes after slow freezing or vitrification. J Assist Reprod Genet. 2005;22:119–27.
17. Cai XY, Chen GA, Lian Y, Zheng XY, Peng HM. Cryoloop vitrification of rabbit oocytes. Human Reprod. 2005;20:1969–74.
18. Tharasanit T, Colleoni S, Galli C, Colenbrander B, Stout TAE. Protective effects of the cumulus-corona radiata complex during vitrification of horse oocytes. Reproduction. 2009;137:391.
19. Tharasanit T, Yamaji YF, Kleinhans FW, Magosaburo K, Colenbrander B, Stout TAE. Effect of maturation stage at cryopreservation on post-thaw cytoskeleton quality and fertilizability of equine oocytes. Mol Reprod Dev. 2006;73:627–37.
20. Aono N, Naganuma T, Abe Y, Hara K, Sasada H, Sato E, et al. Successful production of blastocysts following ultrarapid vitrification with step-wise equilibriation of germinal vesicle-stage mouse oocytes. J Reprod Dev. 2003;49(6):501–6.
21. Aono N, Abe Y, Hara K, Sasada H, Sato E, Yoshida H. Production of live offspring from mouse germinal vesicle-stage oocytes vitrified by a modified step-wise method, SWEID. Fertil Steril. 2005;84:1078–82.
22. Zhang X, Li XH, Ma X, Wang ZH, Lu S, Guo YL. Redox-induced apoptosis of human oocytes in resting follicles in vitro. J Soc Gynecol Investig. 2006;13:451–8.
23. Rall WF. Factors affecting the survival of mouse embryos cryopreserved by vitrification. Cryobiology. 1987;24:387–402.
24. Mahmoudi R, Ragardi Kashani I, Abbasi M, Amidi F, Sobhani A, Abolhasani F, et al. In vitro maturation and fertilization capacity of mouse GVStage oocyte following stepwise vitrification. JCDR. 2008;2:1234–9.
25. Gook DA, Edgar DH. Human oocyte cryopreservation. Hum Reprod Update. 2007;13:591–605.
26. Mahmodi R, Abbasi M, Amiri I, Ragardi Kashani I, Pasbakhsh P, Saadipour Kh, et al. Cumulus cell role on mouse germinal vesicle oocyte maturation, fertilization, and subsequent embryo development to blastocyst stage in vitro. Yakhteh Medical Journal. 2009;11:299–302.
27. Hurt AE, Landim F, Seidel GE, Squires EL. Vitrification of immature and mature equine and bovine oocytes in an ethylene glycol, ficoll and sucrose solution using open-pulled straws. Theriogenology. 2000;54:119–28.
28. Cetin Y, Bastan A. Cryopreservation of immature bovine oocytes by vitrification in straws. Anim Reprod Sci. 2006;92:29–36.
29. Barnes FL, Damiani P, Looney CR, Duby RT. The meiotic stage affects subsequent development of cooled bovine oocytes. Theriogenology. 1997;47:183.
30. Jee BC, Chen HY, Chian RC, Suh CS, Kim SH, Moon SY. Vitrification of immature mouse oocyte using stepwise equilibration before or after in vitro maturation. Fertil Steril. 2009;92: 1153–7.
31. Rzucidlo SJ, Gibbons J, Stice SL. Comparison by restriction fragment differential display RT-PCR of gene expression pattern in bovine oocytes matured in the presence or absence of fetal calf serum. Mol Reprod Dev. 2001;59:90–6.
32. Hunter AG, Moor RM. Stage-dependent effects of inhibiting ribonucleic acids and protein synthesis on meiotic maturation of bovine oocytes in vitro. J Dairy Sci. 1987;70:1646–51.